The RNA target used in these reactions was the fluorescein-labeled material described in Example 21 (*i.e.*, SEQ ID NO:56). Because the efficiency of incorporation of the fluorescein-12-UTP by the T7 RNA polymerase was not known, the concentration of the RNA was determined by measurement of absorbance at 260 nm, not by fluorescence intensity. Each reaction comprised 5 pmoles of the fluorescein-labeled probe (SEQ ID NO:57) and 10 pmoles of the invader oligonudeotide (SEQ ID NO:58) in a buffer of 10 mM MOPS, pH 7.5 with 150 mM LiCl, 4 mM $MnCl_2$, 0.05% each Tween-20 and Nonidet-P40 and 39 units of RNAsin® (Promega). The amount of target RNA was varied from 1 to 100 fmoles, as indicated below. These components were combined, overlaid with ChillOut® evaporation barrier (MJ Research) and warmed to 50°C; the reactions were started by the addition of either 53 ng of Cleavase® A/G or 5 polymerase units of DNAPTth, to a final reaction volume of 10 µl. After 30 minutes at 50°C, reactions were stopped by the addition of 8 µl of 95% formamide, 10 mM EDTA and 0.02% methyl violet. The unreacted markers in lanes 1 and 2 were diluted in the same total volume (18 µl). The samples were heated to 90°C for 1 minute and 2.5 µl of each of these reactions were resolved by electrophoresis through a 20% denaturing polyacrylamide gel (19:1 cross link) with 7M urea in a buffer of 45 mM Tris-Borate, pH 8.3, 1.4 mM EDTA, and the labeled reaction products were visualized using the FMBIO-100 Image Analyzer (Hitachi), with the resulting imager scans shown in Figure 54.

In Figure 54, lanes 1 and 2 show 5 pmoles of uncut probe and 500 fmoles of untreated RNA, respectively. The probe is the very dark signal near the middle of the panel, while the RNA is the thin line near the top of the panel. These RNAs were transcribed with a 2% substitution of fluorescein-12-UTP for natural UTP in the transcription reaction. The resulting transcript contains 74 U residues, which would give an average of 1.5 fluorescein labels per molecule. With one tenth the molar amount of RNA loaded in lane 2, the signal in lane 2 should be approximately one seventh (0.15X) the fluorescence intensity of the probe in lane 1. Measurements indicated that the intensity was closer to one fortieth, indicating an efficiency of label incorporation of approximately 17%. Because the RNA concentration was verified by

A260 measurement this does not alter the experimental observations below, but it should be noted that the signal from the RNA and the probes does not accurately reflect the relative amounts in the reactions.

The reactions analyzed in lanes 3 through 7 contained 1, 5, 10, 50 and 100 fmoles of target, respectively, with cleavage of the probe accomplished by Cleavase® A/G. The reactions analyzed in lanes 8 through 12 repeated the same array of target amounts, with cleavage of the probe accomplished by DNAPTth. The boxes seen surrounding the product bands show the area of the scan in which the fluorescence was measured for each reaction. The number of fluorescence units detected within each box is indicated below each box; background florescence was also measured.

It can be seen by comparing the detected fluorescence in each lane that the amount of product formed in these 30 minute reactions can be correlated to the amount of target material. The accumulation of product under these conditions is slightly enhanced when DNAPTth is used as the cleavage agent, but the correlation with the amount of target present remains. This demonstrates that the invader assay can be used as a means of measuring the amount of target RNA within a sample.

Comparison of the fluorescence intensity of the input RNA with that of the cleaved product shows that the invader-directed cleavage assay creates signal in excess of the amount of target, so that the signal visible as cleaved probe is far more intense than that representing the target RNA. This further confirms the results described in Example », in which it was demonstrated that each RNA molecule could be used many times.

## EXAMPLE 23

Detection Of DNA By Charge Reversal

The detection of specific targets is achieved in the invader-directed cleavage assay by the cleavage of the probe oligonucleotide. In addition to the methods described in the preceding examples, the cleaved probe may be separated from the uncleaved probe using the charge reversal technique described below. This novel

separation technique is related to the observation that positively charged adducts can affect the electrophoretic behavior of small oligonucleotides because the charge of the adduct is significant relative to charge of the whole complex. Observations of aberrant mobility due to charged adducts have been reported in the literature, but in all cases found, the applications pursued by other scientists have involved making oligonucleotides larger by enzymatic extension. As the negatively charged nucleotides are added on, the positive influence of the adduct is reduced to insignificance. As a result, the effects of positively charged adducts have been dismissed and have received infinitesimal notice in the existing literature.

This observed effect is of particular utility in assays based on the cleavage of DNA molecules. When an oligonucleotide is shortened through the action of a Cleavase® enzyme or other cleavage agent, the positive charge can be made to not only significantly reduce the net negative charge, but to actually override it, effectively "flipping" the net charge of the labeled entity. This reversal of charge allows the products of target-specific cleavage to be partitioned from uncleaved probe by extremely simple means. For example, the products of cleavage can be made to migrate towards a negative electrode placed at any point in a reaction vessel, for focused detection without gel-based electrophoresis. When a slab gel is used, sample wells can be positioned in the center of the gel, so that the cleaved and uncleaved probes can be observed to migrate in opposite directions. Alternatively, a traditional vertical gel can be used, but with the electrodes reversed relative to usual DNA gels (*i.e.*, the positive electrode at the top and the negative electrode at the bottom) so that the cleaved molecules enter the gel, while the uncleaved disperse into the upper reservoir of electrophoresis buffer.

An additional benefit of this type of readout is that the absolute nature of the partition of products from substrates means that an abundance of uncleaved probe can be supplied to drive the hybridization step of the probe-based assay, yet the unconsumed probe can be subtracted from the result to reduce background.

Through the use of multiple positively charged adducts, synthetic molecules can be constructed with sufficient modification that the normally negatively charged strand